Remarks

Claims 6-17 are pending in the application, with 6, 12, 13, and 14 being the independent claims. Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 103

Claims 6-17 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Martinez *et al.*, (*Mol. Gen. Genet. 261*:546 (1999)), in view of both Dhadialla *et al.* (*Annu. Rev. Entomol. 43*:545 (1998)) and Saez *et al.* (*Proc. Natl. Acad. Sci. USA 97*:14512 (2000)), as evidenced by Guan *et al.* (*J. Combinatorial Chem. 2*:297 (2000)) and Michelotti *et al.* (U.S. Patent No. 5,304,572). (Office Action, page 2). Applicants respectfully traverse this rejection.

The Examiner alleges that Martinez *et al.* teach controlled gene expression systems involving chimeric receptors comprising the ligand binding domain of ecdysone receptors (EcRs) and also teach specific, dose-dependent activation in response to the non-steroidal EcR agonist RH5992. (Office Action, pages 4-5). The Examiner further alleges that Saez *et al.* teach ecdysone-regulated gene switches and disclose that a number of non-steroidal small molecules are capable of activating the ecdysone system. (Office Action, page 5). Based on the teachings of both Martinez *et al.* and Saez *et al.*, the Examiner is of the opinion that one of skill in the art would be motivated to search for more efficient, readily available non-steroidal EcR agonists. (Office Action, page 6).

Applicants respectfully disagree. Martinez *et al.* disclose that the non-steroidal EcR agonist RH5992, a diacylhydrazine compound, functions to regulate gene expression in an EcR-based expression system. (Martinez *et al.*, page 549, column 1, first full paragraph). Saez *et al.* tested 21 different diacylhydrazine compounds (including RH5992) in their EcR gene expression system and found that 9 out of the 21 compounds showed some ability to activate gene expression. (Saez *et al.*, page 14514, column 2, second full paragraph). These references, taken alone or together, teach that some (but not all) diacylhydrazine compounds that are agonists of the EcR are also capable of activating an EcR-based gene expression system. The references are absolutely silent regarding any teaching of any other compounds or structures that might be useful either for EcR agonist activity or for gene switch activation. In fact, Saez *et al.* state that, in the absence of crystal structure studies, it is unclear what constitutes potent versus ineffective EcR ligands. (*Id.*, page 14513, column 2). Thus, these two references might, at best, suggest to one of ordinary skill in the art to randomly test other diacylhydrazine compounds for the ability to activate a gene switch, but they provide no motivation or guidance towards any other particular structures or compounds.

The Examiner next alleges that Dhadialla *et al.* disclose the compound 3,5-di-tert-butyl-4-hydroxy-N-isobutylbenzamide (DTBHIB) as an EcR agonist with potency similar to RH-5849, a known diacylhydrazine pesticide. (Office Action, page 6). The Examiner acknowledges that DTBHIB does not contain a ketone group that is a critical element of the claimed compounds. (Office Action, page 6). However, the Examiner is of the opinion that DTBHIB is very similar to the claimed compounds, having the same central core and both having EcR agonist activity, and that it is within the skills of the

artisan to build combinatorial libraries around the central core by routine experimentation. (Office Action, page 6). The Examiner further states that, since building combinatorial libraries is a common procedure in the art for identifying variants with improved activity (as evidenced by Guan *et al.*), one of skill in the art would have been motivated to modify the method of Martinez *et al.* by using DTBHIB or its derivatives because DTBHIB was proven to be an efficient EcR agonist. (Office Action, page 6).

Applicants respectfully disagree. Dhadialla *et al.* provide a discussion of EcR agonists and their use as pesticides. Dhadialla *et al.* disclose the compound DTBHIB and its apparent ability to act as an EcR agonist, but indicate that its ability to act as a pesticide is unknown. The Examiner alleges that it would have been obvious to derivatize DTBHIB into compounds encompassed by the present claims because the structure of DTBHIB is similar to the structure of the compounds of the present claims. Applicants assert that it is improper to use the compounds in the present claims to decide that it would have been obvious to make derivatives of DTBHIB that are encompassed by the present claims. This is hindsight analysis based on the teachings of the present specification and is absolutely prohibited. In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teachings. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). The teaching or suggestion to make the claimed combination must be found in the prior art, not in Applicants' disclosure. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). As stated

by the Federal Circuit in *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351 (Fed. Cir. 2001):

> The genius of invention is often a combination of known elements which in hindsight seems preordained. To prevent hindsight invalidation of patent claims, the law requires some 'teaching, suggestion or reason' to combine cited references. *Gambro Lundia AB v. Baxter Healthcare Corp.*, 110 F.3d 1573, 1579, 42 U.S.P.Q.2D (BNA) 1378, 1383 (Fed. Cir. 1997). When the art in question is relatively simple, as is the case here, the opportunity to judge by hindsight is particularly tempting. Consequently, the tests of whether to combine references need to be applied rigorously. *See In re Dembiczak*, 175 F.3d 994, 999, 50 U.S.P.Q.2D (BNA) 1614, 1617 (Fed. Cir. 1999), limited on other grounds by *In re Gartside*, 203 F.3d 1305, 53 U.S.P.Q.2D (BNA) 1769 (2000) (guarding against falling victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher)."

The proper question, based on the cited art, is whether one of ordinary skill in the art, reading Martinez *et al.*, Saez *et al.*, and Dhadialla *et al.*, would find a teaching, suggestion, or motivation within those references to modify DTBHIB to add a ketone structure with a reasonable expectation that the resulting derivative would be an activator in an EcR-based gene expression system. *See In re Grabiak*, 769 F.2d 729, 731, 226 U.S.P.Q. 870, 871 (Fed. Cir. 1985). The answer to this question, as discussed in detail below, is clearly no.

Applicants assert that it is well known in the art that not all compounds that bind to the EcR ligand binding domain have agonist activity and that not all compounds that have agonist activity are also capable of activating an EcR-based gene switch. This unpredictability is confirmed by Saez *et al.*, who tested 21 different diacylhydrazine compounds with known EcR agonist activity and demonstrated that only 9 out of the 21

compounds showed some ability to activate gene expression. (Saez *et al.*, page 14514, column 2, second full paragraph). While DTBHIB is disclosed in Dhadialla *et al.* to bind to the EcR and perhaps be an EcR agonist, there is no teaching nor any suggestion of the ability of DTBHIB to activate an EcR-based gene switch. Given the inability of one of ordinary skill in the art to predict which EcR agonists are also capable of being gene switch activators, there would be no reasonable expectation of success, and therefore would not be obvious, that DTBHIB would function as a gene switch activator.

Applicants point out that the structure of DTBHIB is not encompassed by the compounds of the present claims. Thus, even if one had tested DTBHIB in the gene expression system of Martinez *et al.* or Saez *et al.*, it would not anticipate or render obvious the present claims. The Examiner alleges that it would have been obvious to create derivatives of DTBHIB base on its central core structure and arrive at the compounds of the present claims. Applicants respectfully disagree. The amidoketone structure of the compounds of the present claims is the core structure of the compounds. Removal of the ketone moiety from the compounds eliminates all activity in the gene expression system, demonstrating that the ketone moiety is a necessary part of the core structure. Thus, DTBHIB does not contain the same central core as the compounds of the present claims as DTBHIB does not contain a ketone moiety. Further, building a combinatorial library around the central core of DTBHIB would not produce the present compounds as the central core of DTBHIB does not contain the essential ketone moiety. Based on the lack of knowledge in the art and unpredictability regarding the structural requirements for EcR agonists and gene switch activators, there would be no reasonable expectation by one of ordinary skill in the art that all or even some combinatorial

derivatives of DTBHIB would act as an EcR agonist, much less as an activator of an EcR-based gene switch. Without a reasonable expectation of success, the claimed methods cannot be considered to be obvious over the cited art. At best, one of ordinary skill in the art might consider it obvious to test DTBHIB in an EcR-based gene switch to see if the compound is capable of activating the gene switch. However, it has long been held that an "obvious to try" or "obvious to experiment" standard is not a proper standard for obviousness. *In re O'Farrell*, 7 USPQ2d 1673, 1680-81 (Fed. Cir. 1988). In the absence of any knowledge regarding the ability of DTBHIB to act as a gene switch activator, it would have been even less obvious to derivatize DTBHIB with the hope of producing gene switch activators.

Guan *et al.* does not make up for any of the deficiencies of Martinez *et al.*, Saez *et al.*, and Dhadialla *et al.*, as Guan *et al.* merely discuss the technique of using a core structure as a scaffold for varying substituents to look for compounds having an activity. Guan *et al.* do not discuss EcR agonists, gene expression systems, DTBHIB, or any factor related to the present claims.

The Examiner further alleges that the compound disclosed in Michelotti *et al.* is very similar in structure to DTBHIB, and even though the compound is a fungicide and not a pesticide, one of skill in the art would have been motivated to use it in the method of Martinez *et al.*

Applicants respectfully disagree. The Examiner has stretched the concept of obviousness far beyond its legal limits. The Examiner first alleges that it would be obvious to use DTBHIB in the expression system of Martinez *et al.* because DTBHIB appears to be an ecdysone agonist. As discussed above, the fact that DTBHIB appears to

act as an EcR agonist provides no expectation that the compound will function as a gene switch activator. The Examiner next goes one step further and alleges that it would be obvious to use the compound of Michelotti *et al.* instead of DTBHIB as a gene switch activator because the compounds have similar structures. This allegation is baseless. There is absolutely no teaching, motivation or suggestion in Michelotti *et al.* that the compounds disclosed therein function as pesticides or have any EcR agonist activity. The only teaching in Michelotti *et al.* is that the compounds act as fungicides and are non-toxic to plants. By the Examiner's reasoning, any compound in the world that is similar to DTBHIB is (1) expected to be an EcR agonist; and (2) therefore expected to function as an activator of EcR-based gene expression systems. As discussed above, both of these expectations are unsupported by the cited art or by the general knowledge in the art. In fact, Mikitani (*Biochem. Biophys. Res. Commun. 227*:427 (1996), attached hereto, as Exhibit A), who first disclosed DTBHIB, also teaches that the very closely related compound 3,5-di-tert-butyl-4-hydroxy-N-isopropylbenzamide, which differs from DTBHIB only by having an isopropyl side chain instead of an isobutyl side chain, does not even bind to the EcR (page 428, first paragraph in Results section). Thus, even slight changes to the structure of DTBHIB can destroy EcR agonist activity. This points out the unreasonable position of the Examiner that the compounds of Michelotti *et al.* would be expected to be EcR agonists because they look somewhat similar to DTBHIB.

Furthermore, a *prima facie* case of obviousness based on close structural similarity between chemical compounds is appropriately made only when the compounds are exceedingly close in structure such that that one would expect the compounds to have similar properties. *See In re Payne*, 606 F.2d 303, 313, 203 U.S.P.Q.

245, 254 (CCPA 1979). The only examples of sufficient structural similarity cited in the M.P.E.P. are position isomers (compounds having the same radicals in physically different positions on the same nucleus) and homologs (compounds differing regularly by the successive addition of the same chemical group, *e.g.*, by -CH_2- groups). *See* M.P.E.P. 2144.09. The compounds disclosed by Michelotti *et al.* and DTBHIB do not share sufficient close structural similarity to have an expectation of similar properties as they are not position isomers or homologs of each other. The same is true for DTBHIB as compared to diacylhydrazine EcR agonists. Thus, a *prima facie* case of obviousness has not been made.

It is respectfully requested that the rejection of claims 6-17 under 35 U.S.C. § 103(a) be withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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EXHIBIT

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 427–432 (1996)
ARTICLE NO. 1524

A New Nonsteroidal Chemical Class of Ligand for the Ecdysteroid Receptor 3, 5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide Shows Apparent Insect Molting Hormone Activities at Molecular and Cellular Levels

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Insect molting hormones, ecdysteroids, found in natural products so far have steroidal structures. However, nonsteroidal ecdysteroid agonists show promise as a new type of insecticide and may be useful as probes for the biochemical study of ecdysteroids. 3, 5-Di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide (DTBHIB), discovered by the combination of the automated ecdysteroid receptor binding assay and the ecdysteroid responsive gene expression assay, showed both ecdysteroid receptor binding activity and induction activities of ecdysteroid responsive genes. Furthermore, the inhibition of the Kc cell proliferation and the induction of the cell process protrusion were observed in the presence of DTBHIB in a dose response manner. A similar compound of DTBHIB, however, could neither bind the receptor nor induce the responsive gene. These results provide strong evidence for the ecdysteroid agonist activity of DTBHIB, which has a novel nonsteroidal chemical structure, and might be a valuable lead chemical for higher potential ecdysteroid agonists. © 1996 Academic Press, Inc.

In insects, molting and metamorphosis are elegantly regulated by two types of hormones; the molting hormone, 20-hydroxyecdysone (20-HE), and the juvenile hormones. For pest insect controls, the two hormone systems are promising as target sites because they have vital roles in insects but not in mammals (1). As the juvenile hormone mimics, a number of chemicals have been discovered and used as insecticides (2). In contrast, only dibenzoyl hydrazines are known as non-steroidal mimic of ecdysteroids (3,4). From induction activity of puffs in the *D. melanogaster* salivary gland polytene chromosome by 20-HE, Ashburner hypothesized the first model of the gene expression activity of the receptor bound steroid hormone (5-7). Ecdysone receptor (EcR) heterodimerized with Usp receptor, both of which are classified as members of the nuclear hormone receptor superfamily, directly binds to ecdysteroid responsive element of *Drosophila hsp27* gene, and activates transcription from adjacent promoter when bound with 20-HE (8-10).

Based on this mode of action of ecdysteroids, the author established both an ecdysteroid receptor binding assay system and an ecdysteroid responsive gene expression assay system (11-13). Kc cells were used as materials in these systems because they were highly sensitive to ecdysteroids and possessing relatively short doubling time in the low fetal bovine serum containing medium compared with other insect cultured cell lines (14). Using these two kinds of assay systems, the author screened a number of chemicals and found out that there are four kinds of activities; ecdysteroid receptor binding activity, expression activities of two ecdyste-

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Abbreviations: 20-HE, 20-hydroxyecdysone; DTBHIB, 3, 5-di-*tert*-butyl-4-hydroxy-N-isobutyl-benzamide; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration, 50%; EcR, ecdysone receptor.

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roid responsive genes, cell proliferation inhibition activity, and cellular morphological change induction activity specific for the ecdysteroid agonist in 3, 5-di-*tert*-butyl-4-hydroxy-N-isobutyl-benzamide (DTBHIB).

MATERIALS AND METHODS

Ecdysteroid receptor binding assay. The ecdysteroid receptor binding assay was performed by the 96-well microplate based automated method (11,13). ^{3}H-ponasterone A (as final concentration to be 1.0×10^{-9} M), Kc cell cytosol and the chemicals of different concentrations dissolved in dimethyl sulfoxide (DMSO) were incubated at 25°C, and receptor non-binding free ^{3}H-ponasterone A was separated by dextran coated charcoal method. Nonspecific binding was defined as dpm bound in the presence of 2.0×10^{-5} M 20-HE and each data was corrected by subtracting the dpm value of non-specific from that of each total binding.

Ecdysteroid responsive gene expression activity. Ecdysteroid responsive gene expression activities of the chemicals were assayed by the system as reported (11,12). In brief, the reporter plasmid pHSP27-LUC, which contains ecdysteroid responsive promoter regions of *Drosophila melanogaster hsp27* gene ligated with the firefly luciferase cDNA, was transiently introduced into Kc cells by the electroporation method. After 24 hours of culture with the chemicals, the cellular extracts were made and the luciferase activity was measured. Protein concentration was determined for correction. Furthermore, the reporter plasmid pDAChE-LUC was constructed by the ligation of ecdysteroid responsive *D. melanogaster* acetylcholinesterase gene upstream region (−1520 to +98) to the firefly luciferase cDNA, and used in a similar transient expression assay as utilizing the pHSP27-LUC plasmid but the culture period for pDAChE-LUC introduced Kc cells was 72 hours (15).

Cell proliferation inhibition and morphological change induction. Nearly confluent Kc cells were diluted with M3 (BF) medium (supplemented with 2% fetal bovine serum) to a density of 1.0×10^6 cells/ml. The cells in 2.5 ml aliquots were incubated both with and without the chemicals in 6 cm diameter tissue culture plastic dishes at 25°C under 5% CO_2. For the cell proliferation inhibition assay, cells adhering to the dish wall were scraped off after 72 hours and the cell density was determined by Bürker-Türk hemocytometer. For the cell morphological change induction assay, the cells were observed and photographed by phase-contrast microscopy after 48 hours and 72 hours of culture, respectively. The cell that had the process elaboration length twice as long as its width was scored as ecdysteroid responding cell.

Chemicals. DTBHIB (or 3, 5-di-*tert*-butyl-4-hydroxy-N-isoproyl-benzamide) was synthesized from 3, 5-di-*tert*-butyl-benzoyl chloride and isobutylamine (or isopropylamine) by a conventional method. 20-HE was purchased from Sigma. All chemicals were dissolved in DMSO as stock solution.

RESULTS

Ecdysteroid receptor binding activity. Ecdysteroid receptor binding activity of DTBHIB (Fig. 1A) was determined as displacement of ^{3}H-ponasterone A binding to the Kc cell cytosol (Fig. 1B). The dose-response curve was a typical sigmoid curve. EC_{50} for DTBHIB was 6.0×10^{-6} M, while EC_{50} for 20-HE was 7.0×10^{-8} M (11,13). However, 3, 5-di-*tert*-butyl-4-hydroxy-N-isoproyl-benzamide that possess similar chemical structure of DTBHIB showed no displacement activity at the concentration of 1.0×10^{-4} M.

Ecdysteroid responsive gene expression activity. Ecdysteroid responsive gene expression activity was evaluated by the transient expression of the reporter gene in presence of the compound. The pHSP27-LUC plasmid DNA which contains the *hsp27* gene regulatory element ligated to firefly luciferase cDNA was introduced into Kc cells by the electroporation method (11,12). Dose-response for DTBHIB is shown in Fig. 2A. At 3.0×10^{-6} M, the small but apparent induction was observed, and at 3.0×10^{-4} M, 15-fold induction was observed. EC_{50} for DTBHIB was 8.0×10^{-6} M. 3, 5-di-*tert*-butyl-4-hydroxy-N-isoproyl-benzamide at 1.0×10^{-4} M showed no further induction of luciferase activity compared with the control.

Also the pDAChE-LUC plasmid, that contained 1.6 kb of acetylcholinesterase gene 5′-upstream region ligated to luciferase cDNA was used for a transient expression assay. In this case, 1.0×10^{-5} M DTBHIB showed a 3.7-fold induction, while 3.9-fold induction was achieved at 1.0×10^{-8} M 20-HE (Fig. 2B).

Inhibition of Kc cell proliferation. The effect of DTBHIB on the Kc cell proliferation was examined (Fig. 3A). Without chemicals, Kc cells harvested at 1.0×10^6 cells/ml density proliferated up to 9.1×10^6 cells/ml after 72 hours of incubation. DTBHIB and 20-HE inhibited

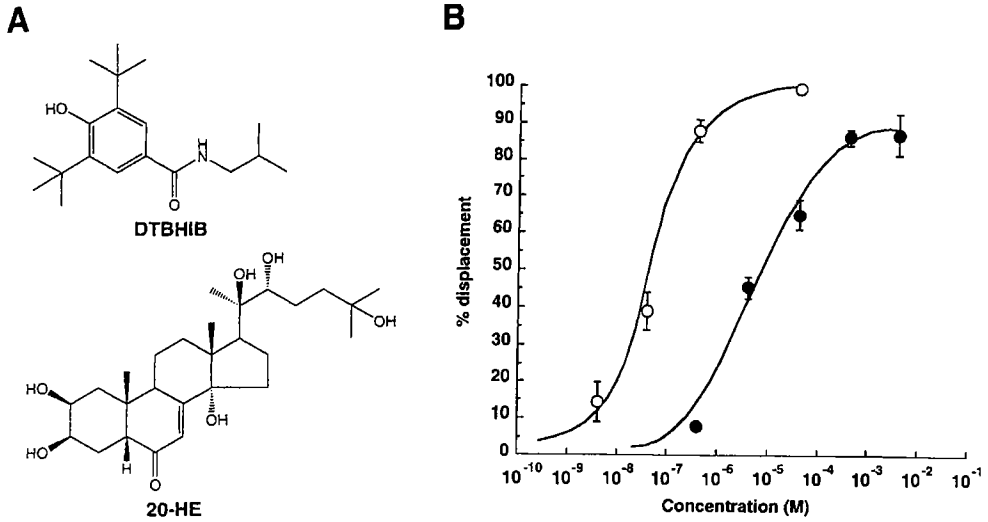

FIG. 1. A: Chemical structure of DTBHIB and 20-HE. **B:** Displacement of ^{3}H-ponasterone A binding to Kc cell cytosol by DTBHIB. Specifically bound ^{3}H-ponasterone A was determined under "MATERIALS AND METHODS" and is plotted as percentage inhibition of ^{3}H-ponasterone A binding versus test concentrations of DTBHIB (●) and 20-HE (○). Each data point represents the mean ± SD (n=3).

the proliferation of the Kc cells and the cell densities were approximately 45% of the control, at 1.0×10^{-5} M and 1.0×10^{-7} M, respectively. EC_{50} for DTBHIB was 3.0×10^{-6} M, while EC_{50} for 20-HE was 7.0×10^{-9} M.

Kc cell morphological change induction. Kc cell shape is spherical when cultured under

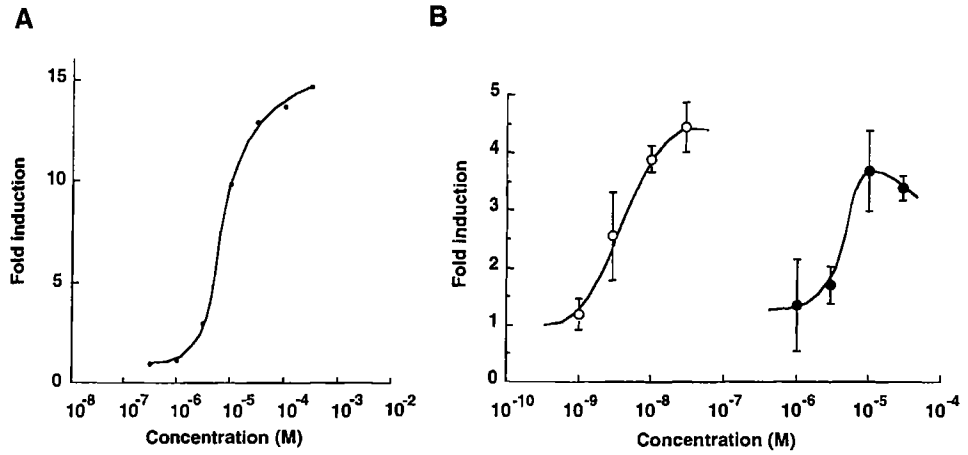

FIG. 2. A: Dose-response curve for luciferase induction activity of DTBHIB against pHSP27-LUC transfected Kc cells. The pHSP27-LUC plasmid was transfected into Kc cells by electroporation method and cultured with and without the chemical. After 24 hours, cell extracts were prepared for the measurement of luciferase activities and protein concentrations (for correction). Each data point is the average of duplicate measurements. **B:** Dose-response curve for luciferase induction activity of DTBHIB against pDAChE-LUC transfected Kc cells. The pDAChE-LUC plasmid was transfected into Kc cells by electroporation method and cultured with DTBHIB (●), 20-HE (○) and only DMSO. After 72 hours, cell extracts were prepared for the measurement of luciferase activities and protein concentrations (for correction). Each data point represents the mean ± SD (n=3).

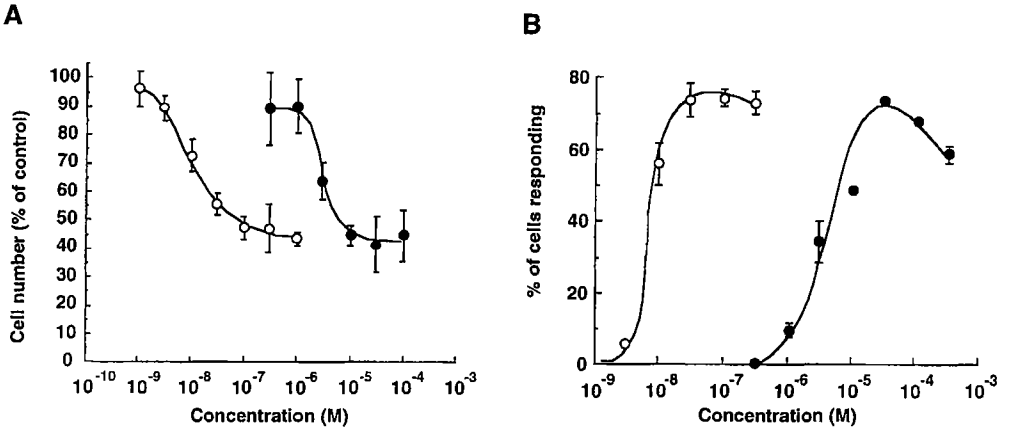

FIG. 3. A: Dose-response curve for Kc cell proliferation inhibition activity of DTBHIB. Kc cells (1.0×10^6 cells/ml) were cultured with DTBHIB (●) and 20-HE (○) for 72 hours. The cell density was determined by hemocytometer. Each data point represents the mean ± SD (n=4). **B:** Dose-response curve for Kc cell process protrusion induction activity of DTBHIB. Nearly confluent Kc cells were cultured with DTBHIB (●) and 20-HE (○) for 48 hours. Ecdysteroid responding cells were scored as having more than twice length of process elaboration compared to shorter width of the cell. Each data point represents the mean ± SD (n=3).

the standard condition. After the addition of DTBHIB to medium, process protruding of cells were observed (Fig 3B). Dose-response was investigated 48 hours after addition of the compound (Fig. 3B). At the concentration of 1.0×10^{-6} M, a slight induction of morphological change was observed by DTBHIB, and at 3.0×10^{-5} M, more than 70% of the cells responded. EC_{50} for DTBHIB was 3.0×10^{-6} M, while EC_{50} for 20-HE was 1.0×10^{-8} M. The Kc cell morphological change induced 72 hours after addition of DTBHIB (2.0×10^{-5} M) and 20-HE (2.0×10^{-6} M) are shown as images of the phase-contrast microscopy (Fig. 4). In both cases,

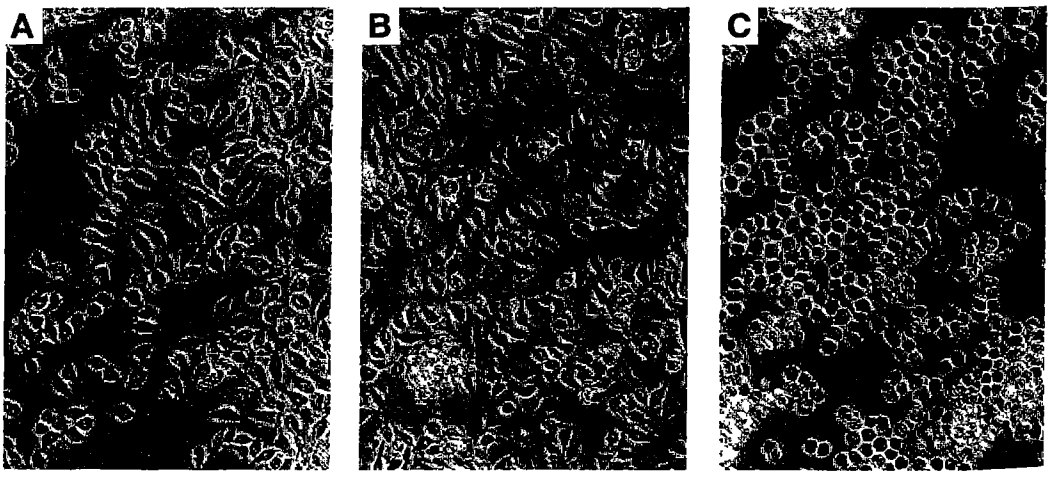

FIG. 4. Kc cell morphological change induced by DTBHIB. Kc cells were cultured with 2.0×10^{-5} M of DTBHIB (A), 2.0×10^{-6} M of 20-HE (B) and only DMSO (C) for 72 hours, and photographed through phase-contrast microscopy (Bar=50 μm).

usually spherical cells showed similar process protruding toward both sides of the cell in response to the chemicals.

DISCUSSION

Based on the mode of action of the hormone, the author screened a number of chemicals for ecdysteroid receptor binding activity as an inhibition of ^{3}H-ponasterone A bound to the Kc cell extract (13). Then the author tried ecdysteroid responsive gene expression assay (12) against two dozens of chemicals that showed receptor binding activity. Among them, DTBHIB possessed the apparent ecdysteroid agonist characters as elucidated in the present study. The ecdysteroid receptor binding potency of DTBHIB was similar to α-ecdysone. Also ecdysteroid responsive gene expression activities of DTBHIB was shown for both *hsp27* gene and acetylcholinesterase gene in transient expression assays. The maximal luciferase fold induction activity of DTBHIB against the pHSP27-LUC plasmid introduced Kc cells (15-fold) was approximately 20% that of 20-HE and α-ecdysone (80-fold; 11,12). EC_{50} of DTBHIB in this gene expression assay was 2 to 3-fold higher than that of α-ecdysone. The pDAChE-LUC plasmid introduced Kc cells also showed induction of luciferase by this compound at 100-fold higher concentration than 20-HE. The cell proliferation inhibition as well as the process protruding induction activity of DTBHIB against the Kc cells were also shown as typical characters of an ecdysteroid agonist. 3, 5-di-*tert*-butyl-4-hydroxy-N-isoproyl-benzamide showed neither the binding activity to ecdysteroid receptors nor the induction activity of an ecdysteroid responsive gene, though having a similar chemical structure to DTBHIB (isopropyl side-chain instead of isobutyl side-chain). Evidences of DTBHIB as ecdysteroid agonist are provided at receptor binding level, specific gene expression level and also at cellular level.

EcR has the highest homology to the vitamin D_3 receptor in their ligand binding region (16). Gene expression activity of ecdysteroid receptor needs heterodimerization with the Usp receptor (9,10) which shows high homology to RXR and some homology to RAR receptor (17). But induction of the ecdysteroid responsive gene was not observed with ligands of the vitamin D_3 receptor (11,12), RXR (10) nor RAR (11,12). A synthetic retinoid agonist Ch55 ((E)-4-[3-(3, 5-di-*tert*-butyl-phenyl)-3-oxo-1-propenyl] benzoic acid) also has 3, 5-di-*tert*-butyl-phenyl- structure (18). However, Ch55 itself had only slight ecdysteroid responsive gene expression activity and was rather cytotoxic at higher concentration (unpublished). These results suggest that the discrimination of chemical ligands among these closely related receptors is very strictly controlled.

As a non-steroidal ecdysteroid agonist, only derivatives of dibenzoyl hydrazines are known so far (3,4). 1, 2-Dibenzoyl-1-*tert*-butylhydrazine showed ecdysteroid receptor binding activity and ecdysteroid responsive gene expression activity as well as cell morphological change induction activity (19). Although the maximal fold induction of luciferase in the pHSP27-LUC plasmid introduced Kc cells with DTBHIB (15-fold) being 1/3 of that with a dibenzoyl hydrazine chemical, RH 5849 (43-fold; 19), DTBHIB showed similar EC_{50} values to RH 5849 in molecular and cellular ecdysteroid assays. As a new chemical class of non-steroidal ecdysteroid agonist, DTBHIB would be an useful ligand in pharmacological characterization of ecdysteroid receptors from various insects as well as discrimination of the ecdysteroid receptor isoforms such as recently found in *D. melanogaster* (20) or subtypes that are not well studied yet. Also, the chemical modifications of this compound may lead to ecdysteroid agonists of higher potency useful as insect growth regulators (IGR) and valuable for insect physiological research.

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